Sylvatic Transmission of Chikungunya Virus among Nonhuman Primates in Myanmar

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Nonhuman primates living in proximity to humans increase risks for sylvatic arbovirus transmission. We collected serum samples from nonhuman primates in Hlawga National Park near Yangon, Myanmar, and detected antibodies against chikungunya (33%) and Japanese encephalitis (4%) viruses. Buffer zones between primate and human communities might reduce cross-species arbovirus transmission.

Several endemic and emerging arboviruses, such as chikungunya (CHIKV), Zika (ZIKV), and dengue (DENV) viruses, have evolutionary origins in nonhuman primates (NHPs) (1,2). These pathogens have adapted sylvatic to urban transmission cycles by using humans as amplifying hosts where NHPs are no longer required for virus maintenance. However, sylvatic arbovirus transmission cycles involving NHPs could act as sources of human infections, which would affect public health. NHPs could enable reemergence of arbovirus infections after immunity has waned following human–mosquito–human transmission. Sylvatic cycles can also provide selective environments where new viral strains can emerge.

CHIKV circulates in distinct enzootic, sylvatic transmission cycles in old world monkeys in the forests of sub-Saharan Africa (2). Limited data are available on sylvatic CHIKV transmission in Asia, but seroconversion has been detected in cynomolgus macaques (*Macaca fascicularis*), pig-tailed macaques (*M. nemistrina*), black-crested Sumatran langurs (*Presbytis melalophos*), and dusky leaf monkeys (*Presbytis obscura*) in Thailand (3,4), and virus has been isolated from long-tailed macaques in Malaysia (5). A sylvatic ZIKV lineage in Africa, infecting Cercopthecidae primate species, is known to circulate widely (6). The only positive ZIKV serology findings in primates in Asia have been in orangutans (*Pongo pygmaeus*) in Borneo, Malaysia, but those exposures were likely from an urban strain (7). Sylvatic DENV cycles occur in the forests of Malaysia in *Macaca* and *Presbytis* spp. monkeys (8) and also in West Africa; sylvatic DENV-2 circulates regularly between *Erythrocebus patas* monkeys and various *Aedes* spp. mosquitoes in Senegal (8). Seroprevalence of Japanese encephalitis virus (JEV) has been reported in cynomolgus monkeys, Japanese macaques (*M. fuscata*), green monkeys (*Chlorocebus sabaeus*), and pig-tailed macaques in several countries in Asia (4,9,10).

Myanmar is among the least studied but most heavily forested region in Asia, and CHIKV, ZIKV, DENV and JEV are highly endemic in humans. We investigated whether Myanmar peri-urban primates, living near the largest urban city of Yangon, are exposed to arboviruses of public health concern and could be sources of spillover or recipients of spillback of human pathogenic arboviral diseases.

The Study

We collected specimens from 107 rhesus monkeys (*Macaca mulata*) and 12 pig-tailed macaques within Hlawga National Park, an open zoo and wildlife sanctuary in Myanmar's Yangon region that covers 6.23 km² (Figure). NHPs are free ranging within this park and have frequent opportunities for human contact.

Serum samples were collected during October 2016–August 2017, which spanned 2 dry/wet seasons. We used a Luminex xMAP multiplex bead-based assay (Luminex Corp., https://www.luminexcorp.com) to simultaneously measure total IgG, IgA, and IgM against CHIKV E1 envelope protein, ZIKV nonstructural protein 1 (NS1), ZIKV envelope protein, DENV-1–4 NS1, JEV NS1, West Nile virus NS1, yellow fever virus NS1, and tickborne encephalitis virus NS1 (Appendix Table, https://wwwnc.cdc.gov/EID/article/28/12/22-0893-App1.pdf). We confirmed positive serum samples by using the plaque reduction neutralization test (Appendix). Conventional reverse transcription PCR targeting conserved regions of *Flavivirus* and *Alphavirus* spp. was performed to detect arbovirus viremia (Appendix).

We identified virus-reactive antibodies among NHPs in Hlawga National Park, suggesting prior exposure to arboviruses, but we did not detect viruses by using PCR, suggesting absence of active infections. We found 33% (39/119) of NHPs were seropositive for CHIKV and 4% (5/119) were seropositive for JEV (Table); all serum samples were negative for ZIKV, West Nile virus, yellow fever virus, and tick-borne encephalitis virus. Using bivariate analysis, we showed specimens collected during the dry season were more likely to be seropositive for CHIKV (p = 0.05). Greater proportions of adult NHPs appeared to be seropositive for CHIKV; however, the difference was not statistically significant. We found no statistically significant associations between sex, age class, or species and specific arbovirus exposure. CHIKV and JEV in NHPs in Myanmar have not been reported, likely because of limited surveillance. Our findings extend the geographic range of potential sylvatic cycles for CHIKV to forests and peri-urban areas of Myanmar.

Our results indicate that NHPs were exposed to CHIKV during a period with no or limited human–mosquito–human transmission, suggesting that seropositive samples resulted from sylvatic exposures. IgG against CHIKV E2 protein can be detected up to 21 months postinfection (*11*). If similar kinetics occur in NHPs and extend to the E1 protein, NHP exposures to CHIKV could have occurred during 2013–2014 or earlier. However, in 2017, we detected CHIKV antibodies in NHPs that were <5 years of age, indicating exposure during an interepidemic period. Human cases of CHIKV were not reported by the Myanmar Ministry of Health during 2011–2018 (*12*), and CHIKV outbreaks are not commonly underreported because a large proportion of infected persons have indicative arthritic manifestations. In 2019, health officials reported widespread outbreaks of CHIKV in Mandalay, Nay Pyi
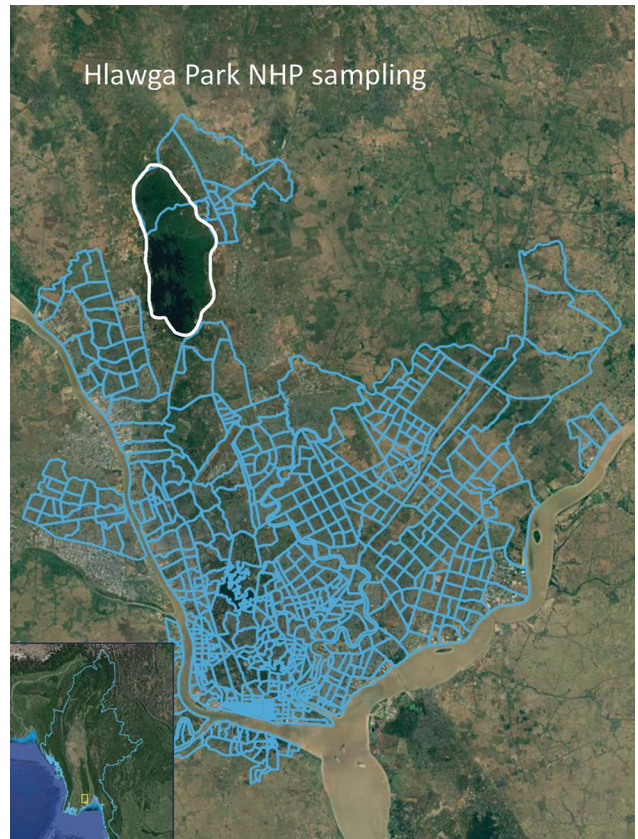

Figure. Hlawga National Park sampling site (white outline) in Yangon in study of sylvatic transmission of chikungunya virus among NHPs in Myanmar. Blue lines show the Yangon city wards south of the park. Inset shows location of Yangon in Myanmar (white box). NHP, nonhuman primate.

Taw, Kachin State, Tanintharyi, and Yangon regions of Myanmar, indicating reemergence of the virus (*12*).

We studied an NHP population that lived in a forested area outside of Yangon and could have played a role in the reemergence of CHIKV in humans. The large proportion of NHPs that were exposed indicated the virus was circulating among sylvatic mosquitos and primates in this park. The absence of reported human infections during the potential period of NHP infection suggested that spillover from humans to NHPs via mosquitoes was unlikely. *Aedes aegypti* and *A. albopictus* mosquitoes, the two primary urban vectors of CHIKV, are also known to feed almost exclusively on humans in the region, providing further evidence that NHP exposures to CHIKV in our study population were of sylvatic origin (*13*).

Our findings indicate that JEV is circulating at the periphery of Yangon, and NHPs can be occasional incidental hosts. JEV is endemic in Myanmar, particularly in the Yangon region (*14*). NHPs are not thought to be potential reservoirs, but are dead-end

Table. Chikungunya and Japanese encephalitis virus prevalence during October 2016–August 2017 in Hlawga National Park in study of sylvatic transmission of chikungunya virus among nonhuman primates in Myanmar*

	Chikungunya virus			Japanese encephalitis virus		
Characteristic	No. positive†	No. negative†	Period prevalence	No. positive†	No. negative†	Period prevalence
Species						
Macaca nemestrina (pig-tailed macaque)	8	4	67 (35–90)	1	11	8 (0–38)
Macaca mulatta (rhesus macaque)	31	76	29 (21–39)	4	103	4 (1–9)
Sex						
M	20	43	32 (21–45)	3	60	5 (0–13)
F	19	37	34 (22–48)	2	54	4 (0–12)
Age class						
Adult	23	35	40 (27–53)	1	57	2 (0–9)
Subadult	16	45	26 (16–39)	4	57	7 (2–16)
Season						
Wet	25	69	27 (18–37)	4	90	4 (1–11)
Dry	14	11	56 (35–76)	1	24	4 (0–20)
Overall	39	80	33 (24–42)	5	114	4 (1–10)

*Values are no. or % (95% binomial exact CI).
†No. positive or negative serum samples according to the total Ig detected by multiplex bead-based assay. Cutoff values were determined according to the lowest median fluorescence intensity value detected by a positive plaque reduction neutralization test.

hosts; they produce a low viremia that cannot subsequently infect mosquitoes (*1*). Low levels of viremia produced in experimental studies and sylvatic cycles involving waterfowl or pigs are well documented. Furthermore, JEV is transmitted to humans by infected *Culex* spp. mosquitoes (most commonly *Culex tritaeniorhynchus*), which feed on many mammals in the region (*15*), making it more plausible that NHPs could be incidental targets of this mosquito species.

We did not confirm NHP exposure to DENV or ZIKV. We identified positive samples by using the Luminex assay, but those samples tested negative when the plaque reduction neutralization assay was used for confirmation. DENV is endemic in humans in Myanmar, and our findings indicate that spillback of urban DENV strains to NHPs is not common in this region or was not detected in our sample size. Given the limited knowledge of the scope of human ZIKV circulation in Myanmar and lack of entomological data, further research is needed to examine potential sylvatic ZIKV cycles among NHPs in Asia.

Conclusions

Our study demonstrates the importance of conducting surveillance of peri-urban primates in regions of high arbovirus transmission and the need for less invasive methods that improve feasibility. Future research on molecular epidemiology of arboviruses in humans, NHPs, and mosquitoes is needed to confirm whether exposures result from potential sylvatic cycles of ongoing transmission or spillback events from urban strains. A heightened awareness of new CHIKV outbreak potential in humans living near NHPs in Hlawga National Park is warranted. Buffer zones between parks and human settlements might reduce future cross-species arbovirus transmission.

Acknowledgments

About the Author

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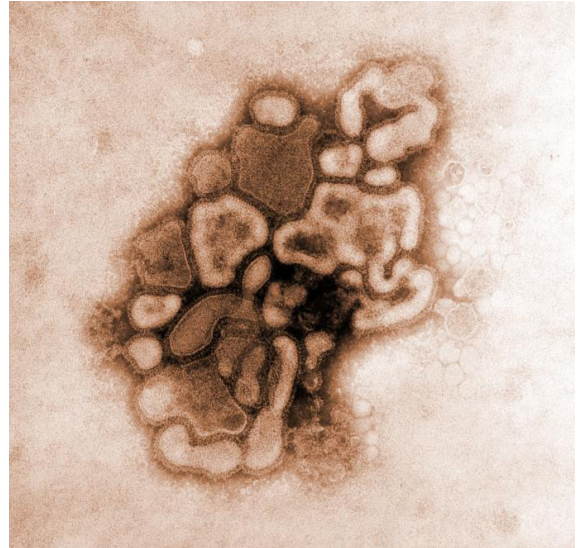
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Appendix

Additional Methods

All animal sampling protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California Davis (approval no. 19300) and the Livestock Breeding and Veterinary Department, Ministry of Agriculture, Livestock and Irrigation, Yangon, Myanmar. We defined the dry season in Myanmar as November through May and the wet season as June through October. We defined a subadult nonhuman primate (NHP) as being fully independent and appearing sexually mature but not fully physically mature (e.g., <full adult size). We defined an adult NHP as having secondary sexual characteristics, adult size, and being sexually mature.

Arbovirus Multiplex Assay

We used the FlexImmArray Arbovirus (19-Plex) NHP IgGMA Antibody Test (Tetracore, Inc., https://www.tetracore.com) to test NHP serum samples. This multiplex bead-based assay uses Luminex xMAP technology and comprises 13 different arbovirus-specific target proteins that detect total IgG, IgA, and IgM antibodies against 10 different viruses (Appendix Table). The test also includes 6 internal controls to monitor assay performance at each step. The multiplex assay uses magnetic microspheres coupled with unique recombinant proteins specific for different viral targets to capture antibodies from NHP serum samples. We mixed 25 µL of each serum sample (diluted 1:250) with 25 µL of antigen-coated microspheres in wells of a 96-well plate. We placed the plates on a shaker set at 800 rpm and incubated for 30 min at room temperature in the dark. The antigen-specific antibodies were immobilized on the microspheres, and we washed unbound material 4× with 200 µL wash buffer. We detected the antigen-captured antibodies by incubating with 25 µL of phycoerythrin-labeled antibodies against NHP IgG, IgA, and IgM conjugates for 30 min at room temperature in the dark on a shaker set at 800 rpm. Unbound material in the plate was washed 4× with 200 µL assay wash buffer. After the final

wash, we resuspended the microspheres in 150 µL of wash buffer and analyzed them by using a Luminex MAGPIX instrument and xPONENT software (Luminex Corp., https://www.luminexcorp.com).

We initially tested serum samples at a 1:250 dilution. Subsequently, we tested suspected positive samples by using serial dilutions of 1:500 and 1:1000. Data were evaluated as the median fluorescence intensity (MFI) of each bead set minus the MFI of the non-specific binding control. To determine initial cutoff values, we evaluated MFIs minus the non-specific binding control for all samples and conservatively set the cutoff values for suspected positives at 3 SD above the mean of the 6 lowest sample values. We confirmed a subset of suspected positive samples identified in the multiplex bead-based assay by using plaque reduction neutralization tests (PRNTs). Cutoff values were subsequently adjusted upwards according to the lowest MFI value at which a positive PRNT was detected.

Plaque Reduction Neutralization Assays

We tested suspected positive serum samples (determined by Luminex xMAP arbovirus assays) for serum neutralizing antibodies by using PRNTs. We heat-inactivated the serum samples at 56°C for 30 min and used the following protocols for each virus.

CHIKV

We used standard plaque methods for CHIKV as previously described (*1*). In brief, we serially diluted serum samples 2-fold in phosphate-buffered saline (PBS) from 1:10 through 1:320 and incubated the samples for 1 h at 37°C with 30 µL of 1600 PFU/mL wild-type CHIKV-06.049 infectious clone that was isolated from a patient in Réunion Island, France (*2*). We grew African green monkey kidney cells (Vero cells) to confluence in 12-well cluster plates, then incubated the cells with 160 µL of serially diluted specimen-virus mixture. After 1 h, we overlayed the cells with 0.5% agarose in Dulbecco's Modified Essential Medium containing 5% fetal bovine serum and incubated for 72 h. We fixed the cells in 4% formaldehyde and removed agarose plugs. We stained the plaques for 10 min with 0.05% w/v crystal violet in 20% ethanol, then washed with cold water. We measured PRNT titers by manually counting plaques according to the serum dilution, where >80% reduction in the number of plaques (PRNT$_{80}$) compared with negative controls was observed. We considered a PRNT$_{80}$ titer of ≥ 10 as positive for CHIKV neutralizing antibodies (*3,4*).

DENV1–4

We used standard plaque methods as described previously with the following modifications. We diluted suspected DENV 1–4-positive serum samples 2-fold in Dulbecco's Modified Essential Medium from 1:10 through 1:320 and incubated the samples for 1 h at 37°C with 60 µL (1:1 ratio) of 100 PFU/µL DENV 1, 2, 3, or 4. We inoculated Vero cells with 100 µL of the serum-virus mixture and incubated for 1 h at 37°C. We overlayed the cells with 1% agarose prepared in 2x solution containing lactalbumin hydrolysate, yeast extract, Earle's Balanced Salt Solution, fetal bovine serum, gentamicin sulfate, fungizone, and 3% sodium bicarbonate. We incubated the cells for 96 h, then added a second overlay of 1% agarose in the 2x solution containing 3% sodium bicarbonate and 3% neutral red and incubated for 24 h at 37°C. We used human convalescent serum samples (BEI Resources, https://www.beiresources.org) as positive controls for DENV 1–4. We used suspected negative NHP serum samples (determined by Luminex xMAP arbovirus assays) as negative controls.

JEV and ZIKV

Standard plaque methods were used as described previously for CHIKV with the following modifications. We diluted serum samples 2-fold from 1:10 through 1:320 in 0.75% bovine serum albumin prepared in PBS. We mixed serum sample dilutions at a 1:1 ratio with 100 PFU of Japanese encephalitis virus strain SA14–14–2 (for JEV) or Zika virus strain PRVABC 59 (for ZIKV) that were diluted in bovine serum albumin in PBS and incubated the mixtures for 1 h at 37°C. We inoculated confluent monolayers of Vero CCL81 cells with 100 µL of each serum-virus mixture in 6-well plates and incubated at 37°C in a 5% CO_2 incubator for 1 h. After incubation, we overlayed the cells with 3 mL 0.6% Oxoid agarose prepared in Minimum Essential Medium containing 4 mmol/L L-glutamine, 50 µg/mL penicillin-streptomycin, 0.25 µg/mL amphotericin B, and 0.01% diethylaminoethyl-dextran. We incubated the plates at 37°C in 5% CO_2 for 3 d, then added a second overlay with 3 mL 1% Oxoid agar containing 0.3% neutral red in Minimum Essential Medium and incubated the cells for an additional 48 h. We included JEV antibody in polyclonal mouse immune ascites fluid as a positive control and a previously tested human serum sample positive for neutralizing antibodies against ZIKV. We also included negative control serum, cell controls, and a virus back-titration to verify virus PFU input. We counted individual plaques with the aid of a lightbox and compared those data with

virus control wells (no neutralizing plasma added). JEV, ZIKV, and JEV mouse immune ascites fluid were kindly provided by the World Reference Center for Emerging Viruses and Arboviruses (https://www.utmb.edu).

PCR Assays for Flaviviruses and Alphaviruses

We processed samples to detect viruses by using consensus PCR, which enables the universal amplification of sequences from viruses within a given family or genus and subsequent discernment of viral strains. We extracted total nucleic acids from whole blood by using Direct-zol RNA Miniprep kits (Zymo Research, https://www.zymoresearch.com) according to the manufacturer's instructions. We reverse transcribed total RNA into cDNA by using SuperScript III (Thermo Fisher, https://www.thermofisher.com) following the manufacturer's instructions and used 2 assays to detect flaviviruses and alphaviruses as described previously (*5*,*6*). As a positive control, we used synthetic DNA constructs containing a T7 promoter sequence that could be transcribed into RNA for reverse transcription-based assays.

Statistical Analyses

To determine associations between NHP demographic and seasonal variables, we evaluated correlations between all variables to assess potential confounding factors. We used chi-square or Fisher exact tests as appropriate to determine associations between specific arbovirus exposure and demographic and seasonal variables. We considered $p<0.05$ to be statistically significant. We then used multivariable logistic regression to assess the association between demographic and seasonal factors that were significant by bivariate analysis. We included variables if they improved model fit according to the likelihood ratio test ($p<0.1$) and minimized the Akaike information criterion. We assessed overall model fit by using the Hosmer-Lemeshow goodness-of-fit test. We performed all statistical analyses by using R, version 3.6.1 (The R Project for Statistical Computing, https://www.r-project.org).

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Appendix Table. Details of the 19-plex microsphere mixture used in the arbovirus multiplex assay in study of sylvatic transmission of chikungunya virus among nonhuman primates in Myanmar*

Analyte no.	Microsphere region	Target antigen
1	26	Zika NS1, antigen 1
2	28	Zika NS1, antigen 2
3	30	Zika Env
4	27	DENV1 NS1
5	35	DENV2 NS1
6	37	DENV3 NS1
7	48	DENV4 NS1
8	39	WNV NS1
9	42	JEV NS1
10	43	YFV NS1
11	44	TBEV NS1
12	45	CHIKV E1, antigen 1
13	46	CHIKV E1, antigen 2
14	47	Instrument control
15	54	Nonspecific binding control
16	51	Human IgM control
17	52	Human IgG control
18	55	Human IgG, IgM, IgA control
19	53	Fluorescent reporter control

*We used the FlexImmArray Arbovirus (19-Plex) NHP IgGMA Antibody Test (Tetracore, Inc., https://www.tetracore.com) to test serum samples from nonhuman primates. CHIKV, chikungunya virus; DENV, dengue virus; E1, envelope protein 1; Env, envelope protein; Ig, immunoglobulin; JEV, Japanese encephalitis virus; NS1, nonstructural protein 1; TBEV, tickborne encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus.